Interactions between Bell pepper endornavirus and acute viruses in bell pepper and effect to the host

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Abstract

Based on the type of relationship with the host, plant viruses can be grouped as acute or persistent. Acute viruses are well studied and cause disease. In contrast, persistent viruses do not appear to affect the phenotype of the host. The genus *Endornavirus* contains persistent viruses that infect plants without causing visible symptoms. Infections by endornaviruses have been reported in many economically important crops, such as avocado, barley, common bean, melon, pepper, and rice. However, little is known about the effect they have on their plant hosts. The long term objective of the proposed project is to elucidate the nature of the symbiotic interaction between *Bell pepper endornavirus* (BPEV) and its host. The specific objectives include: a) to evaluate the phenotype and fruit yield of endornavirus-free and endornavirus-infected bell pepper near-isogenic lines under greenhouse conditions; b) to conduct gene expression studies using endornavirus-free and endornavirus-infected bell pepper near-isogenic lines; and c) to study the interactions between acute viruses, *Cucumber mosaic virus Potato virus Y, Pepper yellow leaf curl virus,* and *Tobacco etch virus* and *Bell pepper endornavirus*. It is likely that BPEV in bell pepper is in a mutualistic relationship with the plant and provide protection to unknown biotic or abiotic agents. Nevertheless, it is also possible that the endornavirus could interact synergistically with acute viruses and indirectly or directly cause harmful effects. In any case, the information that will be obtained with this investigation is relevant to BARD's mission since it is related to the protection of plants against biotic stresses.

Summary Sheet
Publication Summary

PubType	IS only	Joint	US only
Abstract - Poster	0	0	1
Submitted	0	1	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
M.Sc. Student	Escalante	Cesar	Louisiana State University	USA
Ph.D. Student	Khankhum	Surasak	Louisiana State University	USA

Contribution of Collaboration.

The development of near-isogenic lines by the USA principal investigator and the bioinformatics expertise and facilities of the Israeli PIs were essential to accomplish the objectives of the project.

1. Research on molecular biology using RNA nucleotide sequence information relies extensively on bioinformatics. In this area, the project benefited from the expertise provided by Dr. Noa Sela. RNA sequencing was conducted in both, USA and Israel and analyzed data distributed between the two groups. Some sequence data is currently being analyzed and will provide further information to be used in future research proposals.

2. After completing the development of the bell pepper near-isogenic lines, the USA principal investigator (PI) provided the seeds to PI's in Israel for the comparative studies. Simultaneous but complementary experiments were conducted by both groups and results shared by email.

3. The USA PI and one Israeli PI (Dr. Noa Sela) met on September 2, 2015 at the 5th Conference of the International Working Group Legume and Vegetable Viruses in The Netherlands where they discussed research results and exchange data. This meeting helped to conduct an efficient analysis of the data from both groups.

Major Achievements:

One of the most significant finding of this investigation is the evidence that endornaviruses do not appear to be involved in synergistic reactions with acute plant viruses. On the contrary, they may be involved in plant protection against these viruses. Specific accomplishments are:

1. The development of two near-isogenic lines of the bell pepper cv Marengo; one infected with *Bell pepper endornavirus* (BPEV+) and the other one free of *Bell pepper endornavirus* (BPEV-). Comparative studies revealed that the two bell pepper near-isogenic lines were morphologically undistinguishable and that there were no significant differences on fruit yield.

2. Inoculations of the two bell pepper near-isogenic lines with three acute plant viruses did not result in differential symptoms expression. This suggests a lack of synergism between *Bell pepper endornavirus* and acute viruses. However, analysis of the RNA sequence data revealed variations in gene expression between the two bell pepper lines; suggesting virus-specific molecular interactions between acute viruses and *Bell pepper endornavirus*.

3. Results of the biological interaction studies between the plant (bell pepper), *Bell pepper endornavirus* and the acute virus *Pepper mild mottle virus* (PMMoV) showed a differential response. When inoculated with PMMoV, the BPEV+ line yielded lower amount of virus (PMMoV) than the BPEV- line. Moreover, the BPEV+ line reacted with mosaic while the BPEV- line reacted with mosaic and severe systemic necrosis. This suggests that *Bell pepper endornavirus* enhance the plant defense response to PMMoV by helping the plant to avoid the systemic necrotic reaction.

Overall, the achievements of this investigation have advanced our knowledge on plant endornaviruses and will serve as foundation for future research on their potential applications in plant protection strategies.

Changes to original research plan:

There were no major changes of the original research plan. There were only minor changes which included the use of different acute viruses and replacing real-time PCR experiments with RNA sequencing.

Because of a delay in the development of bell pepper near-isogenic lines, a six months no-cost extension of the grant was requested.

Publications for Project US-4725-14

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Submitted	Other	*Cesar Escalante, Noa Sela, Aviv Dombrovsky, and Rodrigo Valverde*	Interactions between bell pepper endornavirus, bell pepper, and acute plant viruses	***Proceedings, Capsicum and Eggplant Conference, 2016***	: 2016	Joint
Published	Abstract - Poster	*Surasak Khankhum, Cesar Escalante, Rodrigo Valverde*	Interaction between persistent viruses of common bean and pepper and four acute viruses.	***Proceedings, Phytobiomes 2015June 30 – July 2, 2015 Washington, DC***	: Page 25 2015	US only

Appendix

1. Publication:

Interactions between *bell pepper endornavirus*, bell pepper, and acute plant viruses

Cesar Escalante[1], Noa Sela[2], Aviv Dombrovsky[2], and Rodrigo Valverde[1]

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Keywords: Capsicum, persistent viruses, acute viruses, RNAseq

Abstract

Persistent plant viruses do not cause detectable symptoms in their plant hosts. In contrast, acute viruses cause symptoms and, in most cases, disease. Most bell pepper (*Capsicum annuum*) cultivars are infected with the persistent virus *Bell pepper endornavirus* (BPEV). To study possible interactions between endornaviruses, the host, and acute viruses, we developed two near isogenic lines of the bell pepper cultivar Marengo: one BPEV-infected and the other BPEV-free. Some agronomic characteristics of the two lines were evaluated under greenhouse condition. The two lines were mechanically inoculated with *Pepper mild mottle virus* (PMMoV), *Potato virus Y*, *Tomato spotted wild virus* (TSWV), and *Cucumber mosaic virus*. Symptoms were recorded and relative amounts of PMMoV and TSWV were determined by ELISA. Preliminary studies on differential gene expression were conducted using RNAseq data. Overall, there were no significant differences in fruit yield and plant phenotype. After acute virus inoculations, there were no differential symptoms between the two lines. Results of the ELISA test showed that the BPEV-infected line yielded consistently less PMMoV than the BPEV-free line; however, the differences were not statistically significant. Results obtained in this investigation suggest that at the symptom level, limited interactions take place between endornaviruses and acute viruses. However, at the molecular level, interactions were obtained.

Introduction

Peppers are cultivated throughout the world for their nutritional and cooking condiment value (DeWitt and Bosland, 1996). *Capsicum annuum* is the specie most widely cultivated. Diseases caused by acute viruses such as *Cucumber mosaic virus* (CMV), *Pepper mild mottle virus* (PMMoV), *Potato virus Y* (PVY), *Tobacco etch virus*, *Tomato spotted wild virus* (TSWV), and several begomoviruses are a limiting factor in pepper production in many parts of the world (Black *et al*., 1991).

The family *Endornaviridae* includes linear RNA viruses that infect plants, fungi, and oomycetes. Their genome ranges from 10-17.6 kb, lack coat protein, and in plants do not cause detectable symptoms (Fukuhara and Moriyama 2008). Plant endornaviruses (*Endornaviridae*) are persistent RNA viruses that do not cause detectable symptoms. In contrast, acute RNA viruses cause symptoms and in most cases disease. Endornaviruses have been reported to infect economically important crops including avocado (Villanueva *et al*., 2012), barley (Candresse *et al*., 2016), bell pepper (Okada *et al*., 2011), common bean (Okada et al., 2013) fava bean (Pfeiffer, 1998), melon (Sabanadzovic *et al*., 2016), and rice (Fukuhara and Moriyama, 2008).

Most bell peppers (*C. annuum*) cultivars and other capsicum species are found infected with the well characterized endornavirus: *Bell pepper endornavirus* (BPEV) and related strains (Okada *et a*l., 2011; Jo *et al*., 2016). *Bell pepper endornavirus* (BPEV), a persistent virus of pepper, has a linear genome of 14,727 bp and contains a single, long ORF encoding a 4815 aa protein (Okada *et al*., 2011). The virus was detected in all bell pepper cultivars tested and transmitted through seed but not by graft inoculations. RT-PCR using degenerate primers revealed variants of BPEV, or closely related species, infecting other *C. annuum* genotypes and three other *Capsicum* species (*C. baccatum, C. chinense* and *C. frutescens*) (Okada *et al*., 2011).

Little is known about the effects that endornaviruses have on plants. The results of testing for the presence of endornaviruses in various crop cultivars suggest that endornaviruses have been introduced into most cultivars of melon, and pepper (Okada *et al*., 2011, 2013; Sabanadzovic *et al*., 2011). In the case of endornavirus infecting melon and bell pepper, all tested cultivars of these two crops were infected (Okada *et al*., 2013; Sabanadzovic *et al*., 2016). Therefore, it appears that during the development of bell pepper and melon cultivars, plant breeders, and possibly people involved in earlier domestication of these crops, unaware of the existence of endornaviruses in the germplasm, selected endornavirus-infected genotypes. This could be an indication that the presence of endornaviruses in these crops is beneficial.

Interactions between endornaviruses and plant pathogens such as acute viruses, fungi or bacteria, have not been studied. It is possible that like acute viruses, endornaviruses could affect the host response to infection by any of these pathogens or other biotic agents.

We have conducted preliminary studies on the interactions between BPEV, bell pepper, and selected acute viruses. The objectives of this investigation were: to evaluate the phenotype and fruit yield of endornavirus-free and endornavirus-infected Marengo bell pepper near-isogenic lines under greenhouse conditions; study the interactions of four acute viruses, CMV, PVY, TSWV, and PMMoV with BPEV in BPEV-infected and BPEV-free Marengo bell pepper isogenic lines; and to conduct Next-Generation Sequencing (NGS) of RNA from the two bell pepper lines infected with selected acute viruses.

Materials and methods

Plant materials and viruses. Using the backcross breeding method, we developed two near isogenic lines of the bell pepper cultivar Marengo: one BPEV-infected and the other BPEV-free. These lines were used in all comparative studies. Virus isolates used in this study consisted of local isolates stored as desiccated tissue in the laboratory.

BPEV detection. DsRNA from BPEV-infected plants and from plants inoculated with PMMoV was extracted using the method reported by Valverde *et al.* (1990). Purified dsRNA was analyzed in agarose gel electrophoresis. Alternatively BPEV ssRNA was detected by RT-PCR as described previously (Okada *et al*., 2011).

Planting. Seeds were planted in steam sterilized soil mix. One month after planting, seedlings were transplanted into 6 L plastic pots. Twenty pots (10 for each line), each containing one plant were placed randomly in the greenhouse. Osmocote© (19-6-3) was incorporated during soil mix preparation. The phenotype of the plants, including time of flowering and fruit setting, were evaluated throughout all developmental stages. Mature, fruits from each line were harvested, counted, measured, weighed, and ANOVA performed to determine fruit yield variations.
Virus inoculations. The two near-isogenic lines were mechanically inoculated with PMMov, CMV, TSWV, and PVY to study the host reaction. Viruses were mechanically inoculated using crude sap diluted in phosphate buffer pH 7.2. Based on preliminary inoculation results, together with available information on the dilution end point for these two viruses, a 1:20 dilution of sap extracted from systemically infected leaves (2-week-old infection) of Tabasco pepper was used. Negative controls consisted of mock inoculated plants. At least eight 4-week-old plants of each line were inoculated with each acute virus by rubbing diluted crude sap onto carborundum-dusted primary leaves. Plants were kept in a greenhouse and symptoms recorded during the first 6 weeks. One and two weeks after inoculation, 1.0 g samples were taken for ELISA testing. RNA was extracted two weeks after inoculation using Qiagen's RNeasy Plant Mini Kit. RNA was quantified, quality assessed and used for RT-PCR testing and NGS. RNAseq libraries were prepared with Truseq protocol and sequenced as single-end with Illumina 2000 at the Technion-Institute of Technology, Israel. Obtained RNA sequences were analyzed using bowtie2 (Langmead and Salzberg, 2012) for RNAseq mapping, RSEM (Li and Dewey, 2011 for transcription quntification and egdeR package (Robinson et al., 2010) for statistical analysis of the data.

Results and discussion

The phenotype of the two near isogenic lines of bell pepper Marengo was similar (Figure 1).

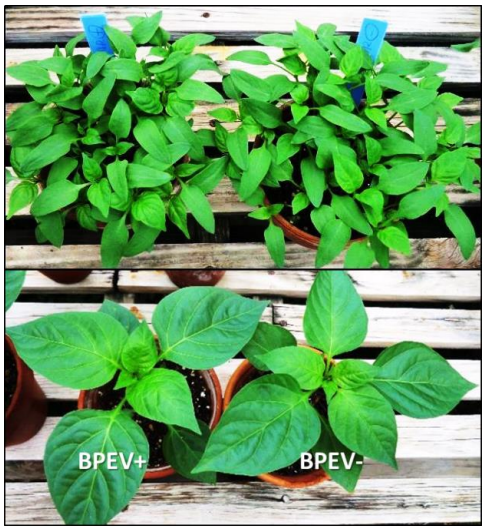

Figure 1. Marengo bell pepper lines, one infected with BPEV (BPEV+) and the other BPEV-free (BPE-).

The plant height, total fresh weight, fruits per plant and total fruit weight was higher for BPEV- than for BPEV+, although, not significant in the case of total fruit weight (Figures 2 and 3).

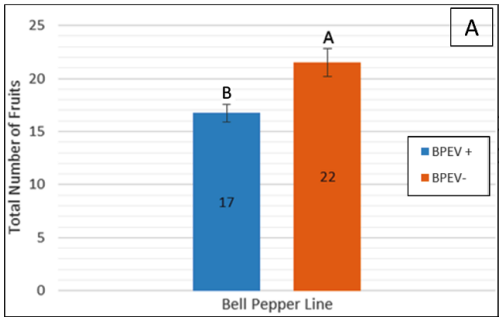

Figure 2. Total number of fruits obtained from two near-isogenic bell pepper lines, one infected with BPEV (BPEV+) and the other BPEV-free (BPE-).

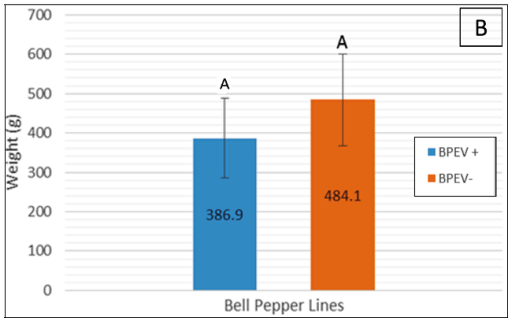

Figure 3. Total fruit weight obtained from two near-isogenic bell pepper lines, one infected with BPEV (BPEV+) and the other BPEV-free (BPEV-).

Biological interactions between CMV, PVY, TSWV, and BPEV were limited and in most cases symptoms caused by these viruses on the two Marengo bell pepper lines were undistinguishable (Figure 4).

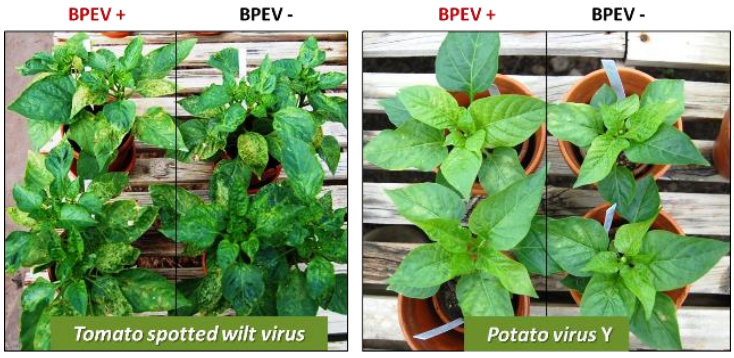

Figure 4. Symptoms on two bell pepper near-isogenic lines, one infected with BPEV (BPEV+) and the other BPEV-free (BPE-) after inoculation with TSWV and PVY.

ELISA results for PMMoV infections showed a difference (Figure 5). The BPEV + line yielded lower readings than the BPEV- lines. Nevertheless, the difference was not significant. DsRNA yields of both, BPEV and PMMoV were not affected.

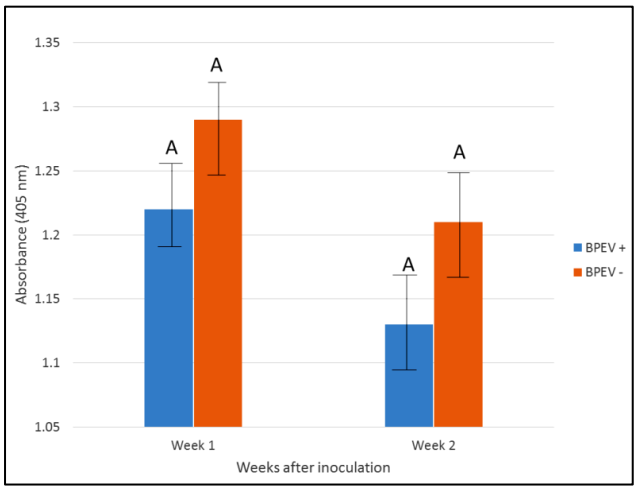

Figure 5. Results of ELISA testing of two bell pepper near-isogenic lines, one infected with BPEV (BPEV+) and the other BPEV-free (BPE-) after inoculation with PMMoV.

Preliminary results of the RNA sequence analyses revealed variations in gene expression between the two bell pepper lines suggesting molecular interactions between BPEV and the host. Transcriptome analysis of peppers infected with PVY, TSWV, or CMV with or without the presence of BPEV revealed various levels of differential gene expression and that the plant response to infection is virus specific (Figures 6 and 7). We plan to further characterize the mechanism underlying the difference in the response of the two bell pepper lines to infection by these viruses.

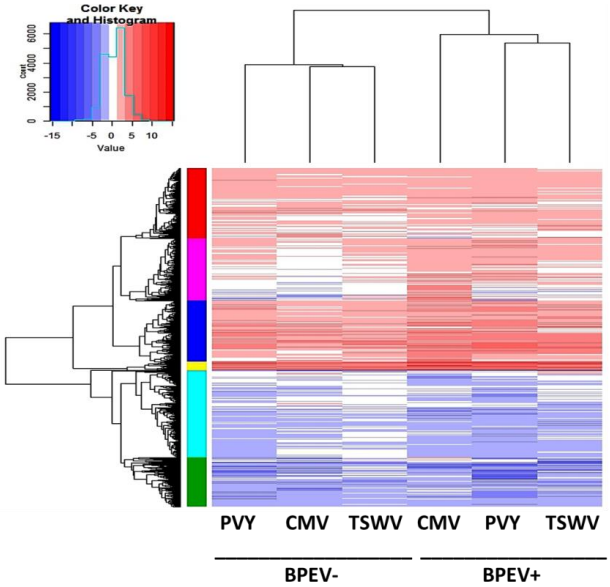

Figure 6. Heat map showing differential gene expression of the various sample treatments.

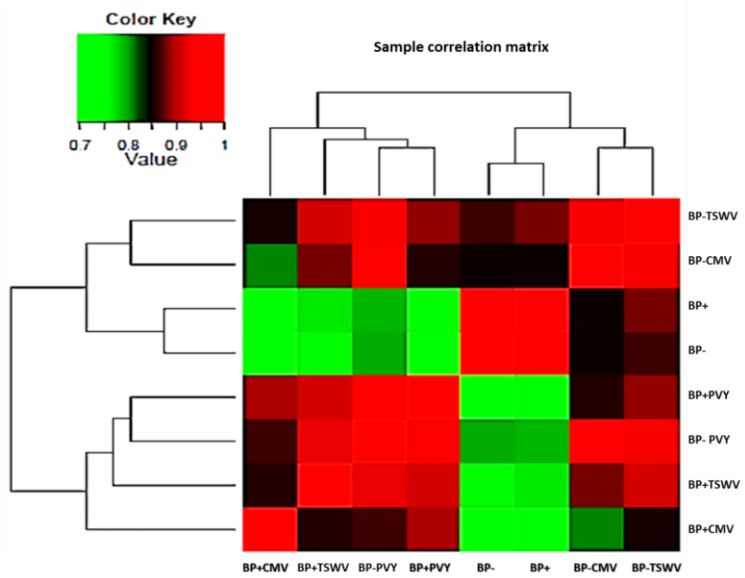

Figure 7. Clustering using a correlation matrix resulting from the comparison of the transcript expression values. BP-=BPEV free line; BP+=BPEV infected line.

Acknowledgments

Funding for this research was provided by a Feasibility Grant (US-4725-14 F) from the US-Israel Binational Agricultural Research and Development Fund (BARD).

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2. Additional data:

Our preliminary RNA sequencing and sequence analyses indicate that in the case of the BP+ line, there are more differential genes expressed in response to acute virus infection than in the BP- line. We intend to further characterize the mechanism underlying the difference in plant response to infection by these viruses.

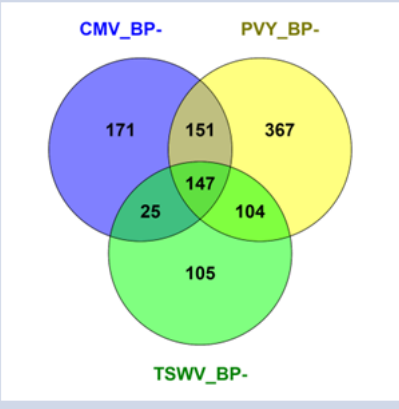
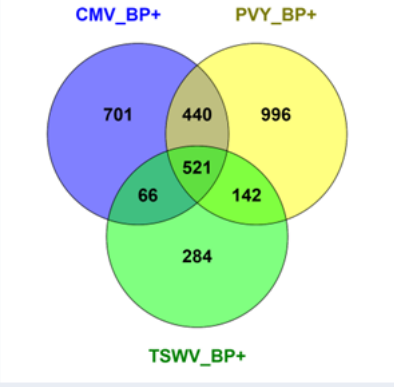
Sample versus healthy	# Diff genes	Venn
BP- CMV	494	
BP- PVY	769	
BP- TSWV	381	
BP+ CMV	1728	
BP+ PVY	2099	
BP+ TSWV	1013	

Figure 1. Venn diagrams for the number of genes expressed for each virus. The diagrams show the overlap of the expressed genes (common genes) for each virus on the two lines.

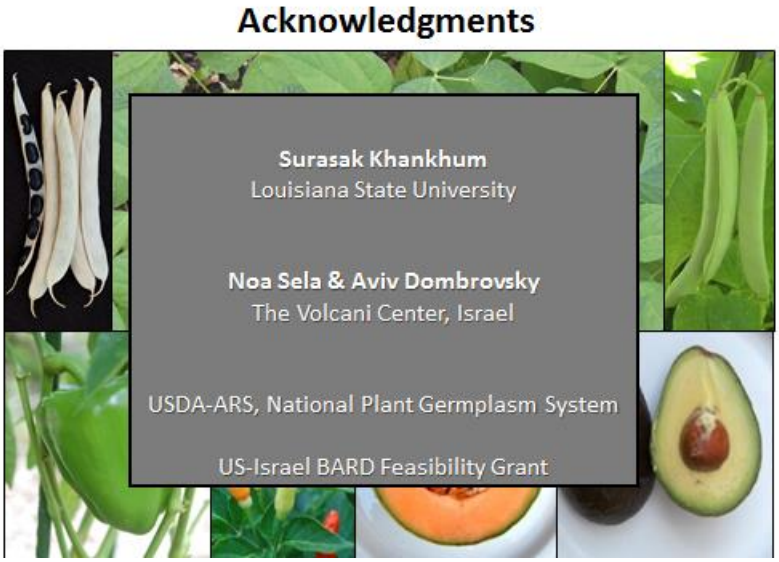
CMV= Cucumber mosaic virus; PVY= Potato virus Y; TSWV=Tomato spotted wilt virus BP+=bell pepper line infected with Bell pepper endornavirus; BP-=Bell pepper line endornavirus-free.

3. Additional paper presentation:

Mr. Cesar Escalante, a MSc. student partially supported by the Feasibility BARD grant presented a seminar at Louisiana State University. Mr. Escalante acknowledged the support by BARD.

LSU AgCenter
Plant Pathology and Crop Physiology
Spring Semester 2016
PLHL 7052 Seminar
Cesar Escalante Guardado
Plant Endornaviruses:
Interaction with the Host and Acute Viruses
Wednesday Feb. 24, 2016
A465 LSB 3:30—4:30PM

Acknowledgement seminar slide:



Mr. Escalante will be presenting a paper at the upcoming 2016 Annual Meeting of the American Phytopathological Society in Tampa, Florida and he will be acknowledging the BARD support.